An Investigation of the Possibility of Multiple Steady States in Continuous Culture of *Escherichia Coli*

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BY
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CERTIFICATE

It is certified that this work has been carried out under my supervision and that this has not been submitted elsewhere for a degree.

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CONTENTS

CHAPTER - I

INTRODUCTION

A culture of microorganisms consists of a great number of cells asynchronously dividing and growing at varying individual rates. The cells increase in mass by growth and in number by reproduction of individual cells when appropriate nutritional and environmental requirements have been met. Growth may be regarded as increase in the cellular substance by replication of each its constituents as a result of interaction with each other and ~~of its const-~~ with the environment. During growth the cell divides and this process is known as reproduction. The environment includes the medium consisting of several essential components and the end products of the metabolism.

A continuous culture is a system in which nutrient medium is continuously fed to the culture in a well-stirred vessel, generally known as a chemostat in the microbiological literature, and simultaneously withdrawing the culture at the same rate at which substrate is fed. The concentrations of all the components, cells, substrates and products are identical in the whole cultivation volume and in the outflowing fluid. The cells are lost from the chemostat by washout and this loss is compensated for by growth and multipication. When the rate of disappearence of cells balances the rate of formation of new cells a steady state is achieved.

A classification of mathematical models of microbial population analysis has been given by Tsuchiya, Fredrickson and Aris (1). In this thesis we shall only be concerned with the nonsegregated model approach which disregards the existence of individual cells and views the population as biomass distributed uniformly throughout the culture. Cell division cannot be considered in nonsegregated models. The mathematical equations of the

nonsegreated models are more amenable to solution under many situations than those of segregated models because of the former's simplicity (2).

The nonsegregated models regard growth as the increase in protoplasmic mass by conversion of medium and gaseous components to protoplasmic mass and other by-products and energy. Growth which is due to interaction between cellular material and environment is modelled as a set of pseudo-Chemical reactions between proto plasmic mass and environmental mass (3). In biological kinetics several chemical species are lumped into a single entity, for example the biomass consists of a large varity of constituents. The rate expressions can be represented by a set of nonlinear ordinary differential equations

$$\frac{d\underline{x}}{dt} = \underline{R}\ (\underline{x}) \qquad \ldots \qquad \ldots \qquad (1)$$

Where $\underline{x}$ is a vector whose components include the concentration of biomass and those of other species which affect biological growth and death; the components of $\underline{R}$ represent the net rate of formation per unit volume of appropriate entity such as concentration of biomass, substrate and other products during batch growth.

In a continuous flow system equation (1) becomes

$$\frac{d\underline{x}}{dt} = -R(X) + \frac{x_0 - \underline{x}}{\Theta} \qquad \ldots \qquad (2)$$

Where $\underline{x}o$ represents inlet substrate concentration, and Θ is holding time.

The steady state in the culture vessel can be represented by putting time derivatives of equation (2)

equal to zero. The resulting algebraic equations may have more than one solution.

$$\underline{R}\,(\widetilde{\underline{x_i}}) = -(\underline{x_o} - \widetilde{\underline{x_i}})\,/\theta \quad \ldots\ldots \quad (3)$$

Where i = 1,2,... s, where s is the number of solutions of equation (3) and $\widetilde{\underline{x}}_i$ represents the concentration of the i th steady state.

When multiple steady states exist, it is of interest to examine which one of the steady states is attained by the system from a given set of initial conditions. This leads to the concept of stability of steady states. Stability refers to a "local concept", i.e. a steady state is locally stable only if small perturbations about it decay in time to restore the system to the steady state. However large perturbations may lead the system to other steady states or posibly keep the system in a state of perpetual oscillations.

Dynamic behaviour of the continuous fermentation has been studied by several workers. The case in which the specific growth rate isconstant has been analysed by Aiba and Humphrey (4) , the formation and interaction of growth inhibitors by Ramkrishna (3), assuming Monod model for growth limitation by Koga and Humphrey (5), and the case of inhibition of growth by high concentration of rate limiting substrate has been studied by Yano and Koga (6). The analysis of these workers of the models based on their assumptions in most of the cases predicts one non-zero stable steady state. Ramkrishna has suggested the possibility of more than one non-zero stable steady states existing in biological reactors. The aim of this work was to test experimentally this possibility which is based on the following analysis(7)

The suggested model is nonsegreated and unstructured in which the formation and interaction of toxic products, and growth supporting products with viable mass is considered. The microbes during growth consume substrate and give more viable mass and other by products. These by products may be toxic substances which interact with viable mass to give nonviable (dead) cell mass. The by-products may be growth supporting substances which in presence of viable mass produce more viable mass. These growth supporting substances can be intermediate oxidation products, proteins, aminoacids, carbohydrates, etc. Meyrath (8) has reported the existance of stimulatory products and adversely acting products, which are the cause of higher growth rate and higher maximum yield of Aspergillus oryzae when grown from smaller and larger inocula. Later in 1964 Meyrath (9) has reported that in vibrating cultures larger amplitudes of the vibromixer lead to stronger influence of inoculum size on the maximum yield. The author points out that this may be due to larger amplitudes of the vibromixer promoting excretion of stimulatory substances. Early and late lags in growth of Bacterium lactis aerogenes when growth on medium in which ammonium sulphate was the source of nitrogen have been reported by Hinshelwood (10). Early lag phase in growth is explained on the basis of the assumption that stimulatory substances are produced during growth. Hinshelwood (10) has pointed out that late lag can be explained on the basis of "Bios' hypothesis or Chesney's hypothesis. Chensney's hypothesis regards the lag as the time required to recover from the toxic effect of the previous environment. These explanations support the hypothesis that toxic and stimulatory substances are produced during growth. Experimental evidence for the formation of inhibtory products has been found by Pratt and Fong (11) for green algal, Chlorella, and by Pratt (12) for fungi. The formation of inhibitors during growth is also

of solution separated (by filtration or centrifuging) from a fully grown culture and added to a young culture completely removes the lag in growth (10) suggests the presence of some stimulatory intermediate in the solution. Expperimental evidence for the presence of growth activator has been reported by Sahyon (15) for Bacterium coli. Diffusible coenzymes have been postulated by Gale and Stephenson (16) in connection with deaminases, and by Yudkin (17) in connection with lactic acid dehydrogenase of Bacterium coli. The variation of activity reported by Woods and Trim (18) with dilution of deaminases of clostridium welchii supports the presences of diffusible substances in the cell which escape from it in early stages of growth.

The following is a mathematical formulation based on the assumption that microbial growth forms both growth supporting substances and toxic products. The reactions are assumed to be.

$$V + a_s S \longrightarrow 2V + \ldots \qquad \text{(i)}$$

$$V + \ldots \longrightarrow V + a_t' T + a_p' P + \ldots \qquad \text{(ii)}$$

$$V + T \longrightarrow N + T + \ldots \qquad \text{(iii)}$$

$$V + a_p P \longrightarrow 2V + \ldots \qquad \text{(iv)}$$

Where V,T,P and N represent viable mass, toxic substances, growth supporting substances and non viable mass produced during growth. The constants a_s, a_t', a_p' and a_p are stoichiometric constants whose interpretations are readily made (2). Equation (i) represents the growth of fresh viable mass and other by-products by interaction of substrate with viable mass, equation (ii) represents the viable mass V interacting with other by-products (represented by dots) to give toxic substance T and growth supporting substances P.

The interaction of viable mass with toxic product T to give non-viable mass N is represented in equation (iii). The last equation establishes that P supports further growth. The increase of pretoplasmic mass in equation (i) and (iv) is assumed to follow the McKendrick and Pai (19) expression of growth. The formation of T and P in reaction (ii) has been considered a first order process and this is justified by the argument that there may be sufficient metabolic products in the environment so that interaction of these with the environment is limited by the concentration of viable mass.

if
x = concentration of biomass
y = concentration of substrate
z = concentration of inbibiter
p = concentration of product P

Then the differential equations for the batch culture can be represented by (7)

$$\frac{dx}{dt} = \mu\, x\, y - K x z + \mu_1 x_p \qquad (4)$$

$$\frac{dy}{dt} = - a_s \mu\, x\, y \qquad (5)$$

$$\frac{dz}{dt} = a_t' K_1 x \qquad (6)$$

$$\frac{dp}{dt} = - a_p \mu_1 x_p + a_p' K_1 x \qquad (7)$$

where μ, μ_1, K, K_1, are reaction rate constants If $a_t' K_1 = \alpha$ and $a_p' K_1 = \beta$ then these equations for continuous culture are given by

$$\frac{dx}{dt} = \mu\, x\, y - K\, x\, z + \mu_1 x_p - D\, x \qquad (8)$$

$$\frac{dy}{dt} = - a_s \mu\, x\, y + D\, (y_o - y) \qquad (9)$$

$$\frac{dz}{dt} = \alpha x - D z \qquad (10)$$

$$\frac{dp}{dt} = - a_p \mu_1 x p + \beta x - D p \qquad (11)$$

Where D is dilution rate and y_o is concentration of substrate entering the chemostat . The concentrations of V,T,& P in inlet stream are assumed to be zero.

In order to ascertain the number of steady states predicted by this model we follow a procedure similar to that used in chemical reactor analysis (20). We equate the time derivatives of y,z, & p to zero, solve for these variables in terms of x and substitute into the right hand side of equation (8).

Thus $\frac{dy}{dt} = \frac{dz}{dt} = \frac{dp}{dt} = 0$

Solving in terms of x for y, z and p one gets

$$y = \frac{D\, y_o}{(a_s \mu x + D)} \qquad (12)$$

$$z = \frac{\alpha x}{D} \qquad (13)$$

$$p = \frac{\beta x}{(a_p \mu_1 x + D)} \qquad (14)$$

Substituting (12),(13),(14) in (8)

$$\frac{dx}{dt} = \frac{\mu D\, y_o\, x}{(a_s \mu x + D)} - \frac{K \alpha}{D} x^2 + \frac{\mu_1 \beta x^2}{(a_p \mu_1 x + D)} - D x \qquad (15)$$

Let $G_1(x) = \frac{\mu D\, y_o\, x}{(a_s \mu x + D)}$

and $G_2(x) = \frac{(\mu_1 \beta - K \alpha) x^2 - (K \alpha a_p \mu_1 x^3 / D)}{(a_p \mu_1 x + D)}$

Assume that $(\mu_1 \beta - K\alpha) > 0$ (16)

and let $A \equiv (\mu_1 \beta - K\alpha)$

$$B \equiv \frac{K\alpha\, a_p \mu_1}{D} > 0$$

Thus $G_2(x) = \frac{A x^2 - B x^3}{a_p \mu_1 x + D}$ (17)

If proper numerical values can be found for the constants then $G_1(x)$ and $G_2(x)$ can assume the shape as shown in Figure (1)

In figure (1) the straight line OD represents the loss of cells in wash-out. OD is a straight line of slope dilution rate D. The curve OABCD represents the growth of cells in the Chemostat. Wherever the straight line OD intersects the growth curve, the loss is compensated by growth, and an equilibrium is described. It is seen from figure that the washout line OD intersects the growth curve at four points, hence four steady states O,A,C,D, are theoretically possible.

PHYSICAL INTERPRETATION OF MULTIPLE STEADY STATES :

It can be seen from figure (1) that there are four steady states. Two of these steady states (2 & 4) are likely stable if the residence time in chemostat is above the minimum residence time. The steady state (2) may be approached by starting the chemostat from low initial organism concentration. If the chemostat is started from a high concentration of inoculum then the formation of P will be facilitated. This product P is a growth supporting substance and hence could support growth and lead to the higher steady state (4) in the chemostat.

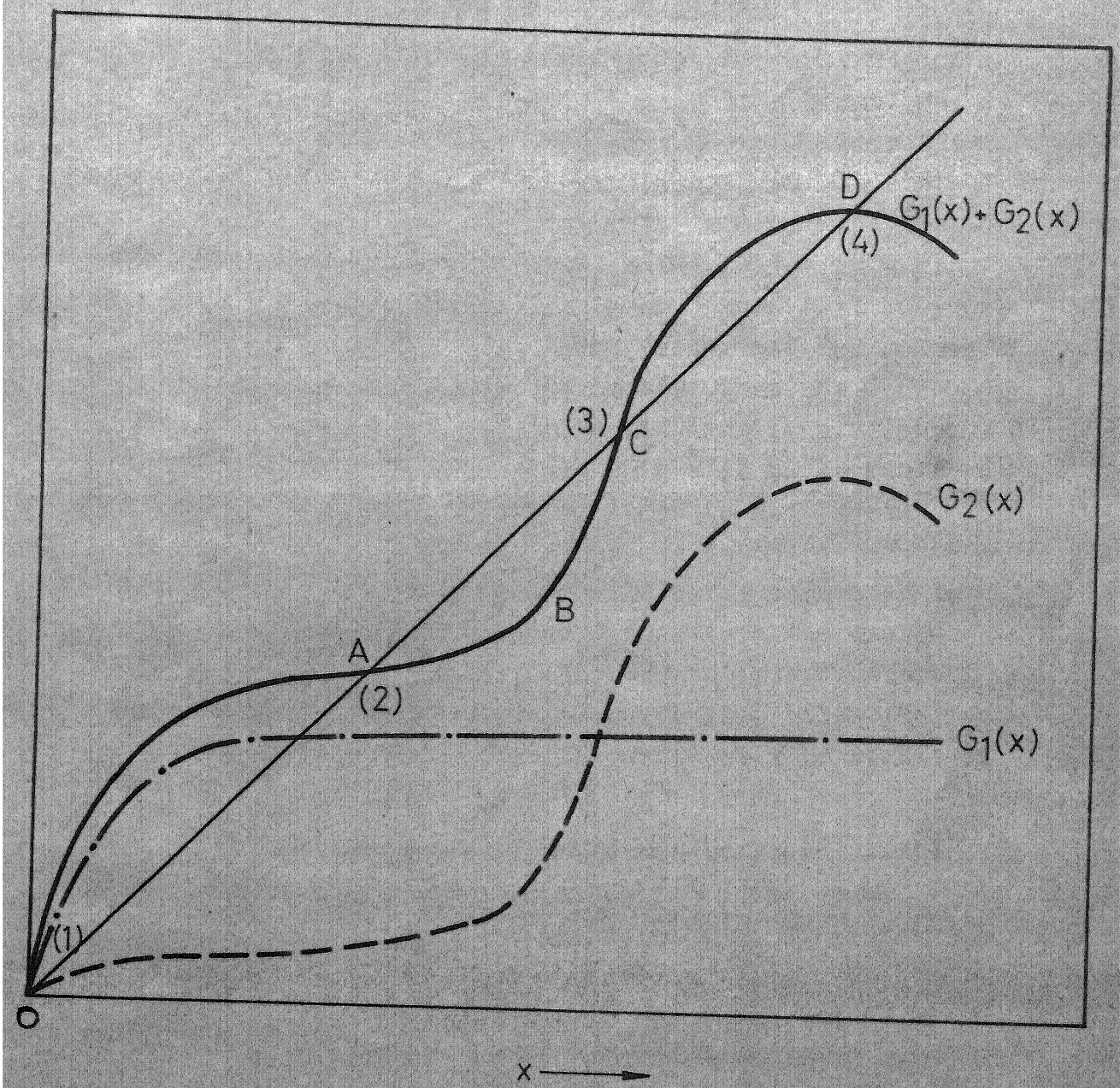

Fig.1 -'G' Curve.

STABILITY AND INSTABILITY OF STEADY STATES :

The stability and instability of steady states can be analyzed by Liapunov's linearization technique. We shall however skip this but infer the stability characteristics from figure (1). The washout steady state is represented by point 0. It can be demonstrated that this is an unstable equilibrium in the following way. It is seen from the figure that near 0 the G curve lies above the washout line which means that for points to the right of 0 the growth rate is more than the washout rate. If the concentration of cells is slightly increased from the equilibrium value then this will be represented by a point right of 0, where there is net growth rate. The concentration of organisms in chemostat will thus go on increasing and will never come back to the original equilibrium value. Therefore the washout steady state 0 is unstable. The washout steady state 0 will be, however, stable if the G curve lies below the washout line, that is washout steady state will be stable if dilution rate is very high.

The steady states (2) and (4) can be shown to be stable. If the concentration of cells is increased slightly above the steady state value then chemostat will return to its original equlibrium value. When the concentration of organisms in the chemostat is higher than the equilibrium value the rate of washout is more then the rate of growth and this is due to the fact that G curve lies below the washout line. This will give a net loss of organisisms and reduce the chemostat to its equlibrium value. If the concentration of cells in chemostat is decreased slightly from its equilibrium value, at this state the G curve is above the washout

line which will give a net growth in chemostat and this will return the chemostat to its original equilibrium value. In this sense the steady state (4) is stable. The same argument can be applied to steady state (2) and it can be shown that steady state (2) is stable. From the same argument as for steady state (1) it can be shown that steady state (3) is unstable. As pointed out by Bilous and Amundson (21) the argument using such a slope condition holds strictly only for instability. For stability it only implies a necessary condition.

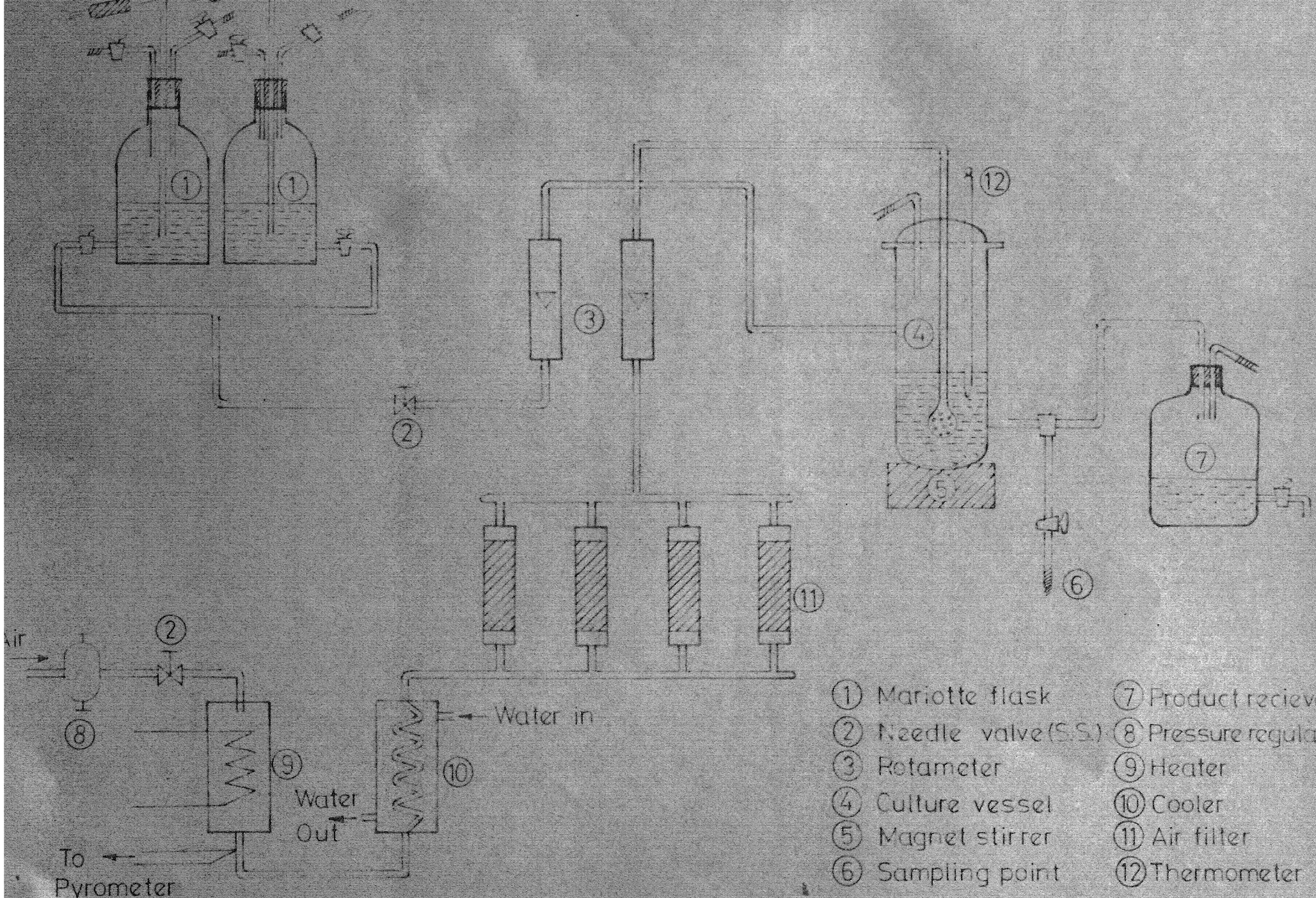

Fig. 2 - Schematic Diagram of Experimental Set-up.

CHAPTER - II

EXPERIMENTAL SET-UP

A. BATCH CULTIVATION :

Batch experiments were carried out in 500 ml. conical flasks. Two shakers placed in the theromostatic chamber whose temperature could be set at any desired value between $20^{\circ}C$ to $40^{\circ}C$ were used for agitation. The shaker speed was preadjusted at a level to give sufficient agitation of the culture in flasks. Care was taken that the culture did not wet the cotton plug. The temperature of the theromostatic chamber was adjusted to $35^{\circ}C$ in each experiment. The flasks were withdrawn from the shaker for analysis at one hour intervals. Duplicate flasks were taken out each time.

B. CONTINUOUS CULTIVATION :

The experimental set-up used in this study was essentially the one used by Gandhi(22) with some modifications. It consisted of the following parts.

1. Dosing Device
2. Culture Vessel
3. Air Sterilizing unit
4. Aeration and agitation device
5. Sampling device

DOSING DEVICE :

The dosing device used in this work was based on the principle of the Mariotte flask(23) serving as the reservoir of the nutrient medium. The liquid flows from feeding bottle under constant hydrostatic pressure. The Mariotte flask consisted of a 5 liter bottle in which three tubes were connected to the mouth of the bottle. Two of these three tubes were provided with stop cock, while the third one was up to the bottom of the flask. Air

entered the flask through this third tube regardless of the receding actual level of the liquid in the flask. This device was placed in a constant temperature room as the system was very sensitive to changes in environmental pressure and temperature. The outflowing stream of the liquid nutrient medium from the storage vessel to the culture vessel was controlled by the use of a stainless steel needle valve. The flow rate of nutrient medium was measured by a glass rotameter (Brooks instrument Co. Hatelfield, Tube size 2-15-3) placed after the needle valve. One stop cock was provided at the outlet of each Mariotte flask. The outlets were connected with each other by using rubber tubing and T-joint. The storage flasks were filled asceptically by sterilized nutrient medium with the help of a Manostat Varistaltic pump.

CULTURE VESSEL :

The culture vessel was made of a 4" diameter pyrex glass tubing, which would enable visual observation of cultivation, moreover a glass vessel is easy to clean and sterilize. The culture vessel was 12 inches in height. The mouth of the culture vessel was made of a standard ground glass joint. The top of the vessel had four inlet tubes of about 1/2 inch in diameter. The outlet for the medium was at about 2 inches from the bottom. The nutrient medium was fed to the culture vessel from the side fitted with a standard [illegible] glass joint at about 3 inches from the top. The Central inlet tube at the top of the culture vessel was used for air inlet. The other three openings at the top were used for air outlet, inoculation and temperature measurement. The air inlet tube, air outlet tube and theromometer were fitted in the culture vessel openings by rubber cork and teflon tape . The air outlet was

a U tube packed with glass wool at the mouth. The end of the air inlet tube in the culture vessel was made in the form of a ½" diameter bulb of pyrex glass with small holes on the bulb for the passage of air.

AIR STERILIZATION UNIT :

A continuous supply of air at constant rate and pressure was available from the compressor of Sanitary Engineering Laboratory. The outlet pressure of the air from the compressor was maintained between 80-100 psig by means of a pressure trip device. A pressure regulator was used to get the air at a constant pressure of 5 psig. A needle valve was placed after the pressure regulator to control the volumetric flow rate of air. The air then was passed through the heater. The heater was provided with a heating element and the voltage was regulated by a variac. An 80 volts supply was used to heat the heater element as this was sufficient to raise the temperature of air to 150°C. The air temperature was measured by a thermocouple at the outlet of heater and connecting it to a pyrometer. The air was then cooled by passing through a helical cooling coil, the coils were cooled by circulating water outside the coils. The air was then filtered by passing through an air filter. The air filter was made of pyrex glass tubing of 1" diameter and 18" long packed with non absorbent cotton. The ends of the tubes (air filters) were closed by ground standard glass joints. Four filters were used in parallel in such a way that each filter was used for air filteration. One filter was used for 12 hours only. The flow rate of air was measured by placing a glass rotameter (Brooks Instrument Co. Hatelfield, Tube size 2-15-3) in the air line after filter and before culture vessel. This arrangement gave sterilized air at constant flow rate, pressure, and temperature.

AERATION AND AGITATION :

It is essential for chemostatic continuous cultivation processes that the culture is homogeneous and the added medium is dispersed equally into all parts of the culture. For a culture vessel of a working volume of 1 liter it was found that aeration is sufficient for the agitation of the liquid culture. At the end of the aeration tube a bulb of ½" diameter with small holes on its surface distributed the air uniformly throughout the culture. This provided simultaneously uniform aeration and efficient agitation. The mixing in the lower part of culture vessel below the aeration bulb was achieved by using a magnetic stirrer. A preliminary experiment was performed in which dissolved oxygen of the culture liquid was measured by using galvanic cell oxygen analyser, and it was found that the air at rate of 3 liters per minute was sufficient for aeration.

SAMPLING :

The sampling port was made near the outflow point of the culture vessel by using a three way stop cock and another two way stopcock near the sampling point. The mouth of the sampling point was heat sterilized before collecting the samples and then samples were collected asceptically. The sampling point was kept in a smpling chamber. The sampling chamber was a chamber made of glass and wood with two 4 inch diameter openings on the sides to insert the hands in to the chamber for collecting the samples asceptically. The end of the sample point was kept closed by sterilized cotton in between withdrawals.

MAINTENANCE OF CONSTANT LEVEL IN THE CULTURE VESSEL :

The constant level in the chemostat was maintained by connecting the outlet tubing of the cultivator to the product receiver by three L joints, as shown in figure 2. The

upper surface of the culture vessel and the product receiver were connected with the surrounding atmosphere by putting glass wool filters in the air outlet lines. This arrangement is necessary for maintaining constant pressure and flow rate of nutrient medium.

MAINTENANCE OF CULTIVATION TEMPERATURE :

The entire continuous cultivation set-up was placed in a constant temperature room. The temperature of the room was maintained constant $\pm$ 3°C by the help of a theromostat arrangement. Although the optimum temperature for the strain of Escherichia Coli standard is 37°C, the temperature was kept at 35°C for all the experiments as it was difficult to work in a room maintained at 37°C.

CHAPTER - III

EXPERIMENTAL METHODS

A. CULTURE MEDIUM :

A chemically defined medium of the following composition was used for the continuous cultivation of E.Coli. Glucose was the sole carbon source and was also the growth limiting component.

- MEDIUM (A) -

Di Ammonium Hydrogen Phosphate $(NH_4)_2HPO4$	2 gms
Potassium Dihydrogen Phosphate (KH2P04)	2 gms
Sodium chloride (NaCl)	4 gms
Magnesium Sulphate ($MgSO4\ 7H_2O$)	.1 gms
Glucose	as below
Distilled water	1 litre

Glucose was added 2 gms per litre for inoculum preparation and 20 mgms per litre and 10 mgms per litre in continuous cultivation of E.coli experiments. The pH value of the nutrient media was adjusted to 7 (before sterilization) by adding 5 N NaOH solution.

The composition of the solid media used for maintaining the culture of E.coli was :

Agar	=	10 gms
Beef extract	=	1.5 gms
Peptone	=	2.5 gms
Distilled water	=	500 ml.

pH was adjusted (7 to 7.5) and the medium was sterilized at 15 psig for 30 minutes.

B. CULTURE :

A strain of Escherichia coli supplied by the National Sugar Institute, Kanpur maintained in the laboratory was used throughout the study. Cell material transferred from an

agar slant was inoculated in 10 ml of medium (A) in test tube and incubated at 35°C for 21 hours. This growth was used as inoculum for the preparation of the seed culture. Two mls of liquid culture from the test tube was transferred asceptically into 200 ml. of liquid medium in 500 ml. conical flask and incubated at 35°C for 18 hours. This culture provided young cells of uniform age ready for use in the chemostat. Occasionally microscopic observations were made to check the purity of the culture.

C. STERILIZATION :

a. Set-Up: The culture vessel, air filters, rotameter and Mariotte flasks with lines connected with them were sterilized in a steam sterilizer at 15 psig. for 30 minutes before each experiment. The air filters were first sterilized and then dried in an oven at 110°C for 15 minutes. In all cases the chemostat and their accessories were removed from the sterilizer, cooled down to room temperature, unpacked from paper wrapping and cotton pads, and were fitted up asceptically.

b. Media :

Small volumes of liquid and solid culture media were sterilized in test tubes and conical flasks in the autoclave twice, at 24 hours interval at 5 Psig for 30 minutes each time. The glucose in the nutrient medium was found to decompose on sterilization. Therefore large volumes of nutrient medium, up to 4 litres, were sterilized by taking 4 litre nutrient media without glucose in 5 litre flask and keeping it in the autoclave at 10 psig for 30 minutes. Glucose solution of known concentration was separately sterilized and known volumes of this sterilized glucose solution was trasferred to give desired concentration of nutrient medium.

D. START-UP OF CONTINUOUS CULTIVATION :

One litre of start-up culture was prepared in the chemostat by batch growth of E.coli for 18 hours at 35°C. The flow of air at controlled rates from the compressed air reservoir through the air filter was started and maintained for about 10 minutes. Sterilized nutrient medium was then allowed to flow through the culture vessel at a particular flow rate. Samples of the culture were then taken hourly and absorbance of the samples were measured. For experiments to be started with low population density of cells, the chemostat was filled by sterilized nutrient medium and was inoculated by 2 ml. of inoculum from the liquid culture grown for 18 hours on nutrient medium (A) (glucose concentration 2 gms per litre). The air was then let into the chemostat for 10 minutes and nutrient medium flow was started and maintained at particular rate.

The culture vessel, connecting lines, two Mariotte flasks with other accessories were steam sterilized in the autoclave at 15 psig. After sterilization they were asceptically connected by connecting tubes. The storage flasks were then filled by sterilized nutrient medium with the help of the Manostat varistaltic pump. The stop cock of one of the storage flasks was kept open at the time of feeding the nutrient medium to the culture vessel. When this storage flask was empty, the stop cock of the other storage vessel was opened while that of the first was closed. The empty storage flask was then filled with nutrient medium by the help of the manostat varistaltic pump. The above operations were repeated when the second storage flask was empty. The particular flow rate could be obtained by adjusting the needle valve placed between the rotameter and T-Joint connecting the two storage flasks.

CHAPTER - IV

RESULTS, CONCLUSIONS AND RECOMMENDATIONS

EXPERIMENTAL RESULTS :

Batch culture experimental results on the growth of E. coli are presented in table(1) with initial glucose concentration of 20 mgm/litre. The growth parameters measured were glucose concentration, viable count, dry weight and absorbance, etc.

Continuous culture experimental results on the growth of E. coli are presented in tables 2 to 7 with feed glucose concentration of 20 mgm/litre and 10 mgm/litre. The results for 20 mgm/litre glucose feed concentration have been obtained for holding times of 3 hours and 1.5 hours. The experiments with 10 mgm/litre of glucose feed concentration were performed for holding time of 3 hours only.

ANALYSIS OF RESULTS :

The experimental data of batch experiments have been plotted in figure (3). The viable count, dry weight and absorbance have been plotted on a semilog graph. The determination of constants from these batch culture experiments is difficult. At low substrate concentration the determination of dry weight and the substrate concentration accurately with time is difficult as the value of these were very small.

The experimental data of continuous cultivation are plotted in figure (4). The results of continuous cultivation are shown in table (8). It is seen that the chemostat shows only one steady state.

CONCLUSIONS:

Based on the results of present study it may be concluded that under the range of initial conditions the system does not show multiple steady states. The explanation for the absence of multiple steady states in the chemostat may be due to one or more of the following.

1. Toxic and growth supporting substances are not produced in 3 hours when feed substrate concentration was 20 mgms/litre, and in 1.5 hours when feed substrate concentration was 10 mgms/litre in chemostat.

2. E.Coli contains 50 % protein and very little % of carbohydrates. The available carbodydrates may not compare favourably with the 10 mgms/litre feed glucose concentration to give a noticable higher steady state.

3. There is lag in growth of E. coli in the chemostat when started from higher biomass concentration so that the cells are washed out during this lag phase without supporting the growth necessary for attaining the higher steady state.

Experiments with very high initial bio mass concentration with longer holding times should be performed. A nutrient medium deficient in nitrogen can be used, so that the 50% protein available in cells of E.coli can be used to support the growth to give higher steady state.

RECOMMENDATIONS :

1. A large number of experiments should be performed under various conditions to test the hypothesis. The experiments with larger holding time of several hours is recommended for future work. Holding time should be long enough so that it is certain that toxic substances and growth supporting substances produced during growth are interacting as assumed in the suggested growth model.

2. The selection of a proper strain for testing a hypothesis is most important. The organism should be such that during growth it produces both types of substances. Continuous cultivation of mold (Aspergillus Oryzae) to test the existence of multiple steady states is recommended for future work, as this has been reported in the literature (7,8) that this produces growth supporting and adversely acting substances during growth. The measurement of growth parameters in case of mold growth is a little difficult. A proper method for measuring growth parameters in continuous cultivation thus should be first worked out.

3. When low glucose substrate concentration is used in continuous cultivation, a method by which very low concentrations of glucose can be measured in solution should be used.

TABLE I

BATCH EXPERIMENT DATA

Sl. No.	Time (hrs)	Absorbance	Dry Weight gms/100ml.	Viable Count x 10^6	Glucose Conc. mgms/lit.
1	0	.009	-	5	20
2	1	.01	-	5.8	12.5
3	2	.015	-	15.4	5
4	3	.04	.001	43	
5	5	.06	.0013	87	
6	6	.06	.0015	115	
7	7.5	.06	.0016	129	
8	8.5	.062	.0015	125	
9	9.5	.062	.0015	115	
10	10.5	.063	.0013	110	
11	12.5	.064	.0015	118	
12	14.5	.063	.0014	100	
13	16.5	.064	.0013	94	
14	20.5	.062	.0015	88	
15	24.5	.064	.0015	153	

T A B L E - II

CONTINUOUS CULTURE DATA

Substrate concentration = 20 mgm/Litre
Holding time = 1.5 hrs.

Sl.No.	Time (Hrs)	Absorbance
1	0	.37
2	1	.21
3	2	.19
4	3	.18
5	4	.17
6	5	.11
7	6	.075
8	7	.06
9	8	.055
10	9	.045
11	10	.046
12	11	.045
13	12	.045
14	13	.045
15	14	.045
16	15	.043
17	16	.045

TABLE - III

CONTINUOUS CULTURE DATA

Substrate concentration = 20 mgm/litre

Holding time = 1.5 hours.

Sl.No.	Time	Absorbance
1.	0	.01
2	1	.015
3	2	.04
4	3	.045
5	4	.045
6	5	.045
7	6	.045
8	7	.045
9	8	.045
10	9	.045

TABLE - IV

CONTINUOUS CULTURE DATA

Substrate concentration = 20 mgm/litre
Holding time = 3 hours

Sl.No.	Time(Hrs)	Absorbance
1	0	.42
2	1	.38
3	2	.36
4	3	.35
5	4	.35
6	5	.31
7	6	.24
8	7	.18
9	8	.14
10	9	.11
11	10	.09
12	11	.08
13	12	.07
14	13	.06
15	14	.055
16	15	.055
17	16	.055
18	17	.055

TABLE - V

CONTINUOUS CULTURE DATA

Substrate concentration = 20 mgm/litre
Holding time = 3 hours.

Sl.No.	Time(Hrs)	Absorbance
1	0	-
2	1	.01
3	2	.015
4	3	.03
5	4	.04
6	5	.05
7	6	.055
8	7	.06
9	8	.055
10	9	.06
11	10	.055
12	11	.055

TABLE - VI

CONTINUOUS CULTIVATION DATA

Substrate concentration = 10 mgm/Litre
Holding time = 3 hours.

Sl.No.	Time(Hrs)	Absorbance
1	0	.45
2	1	.35
3	2	.2
4	3	.17
5	4	.13
6	5	.11
7	6	.09
8	7	.07
9	8	.045
10	9	.04
11	10	.03
12	11	.02
13	12	.015
14	13	.01
15	14	.01
16	15	.015
17	16	.01
18	17	.01

TABLE - VII

CONTINUOUS CULTIVATION DATA

Substrate concentration = 10 mgm/litre
Holding time = 3 hours.

Sl.No.	Time(Hrs)	Absorbance
1	0	0
2	1	.01
3	2	.015
4	3	.01
5	4	.01
6	5	.01
7	6	.01

TABLE - VIII

RESULTS OF CONTINUOUS CULTIVATION

Absorbance					
Feed substrate concentration 20 mgms/litre				10 mgms/litre	
Holding time 1.5 Hrs		3 Hrs.		Holding time 1.5 Hrs.	
Initial Biomass Concentration		Initial biomass concentration		Initial biomass conc.	
High	Low	High	Low	High	Low
.045	.045	.055	.055	.01	.01

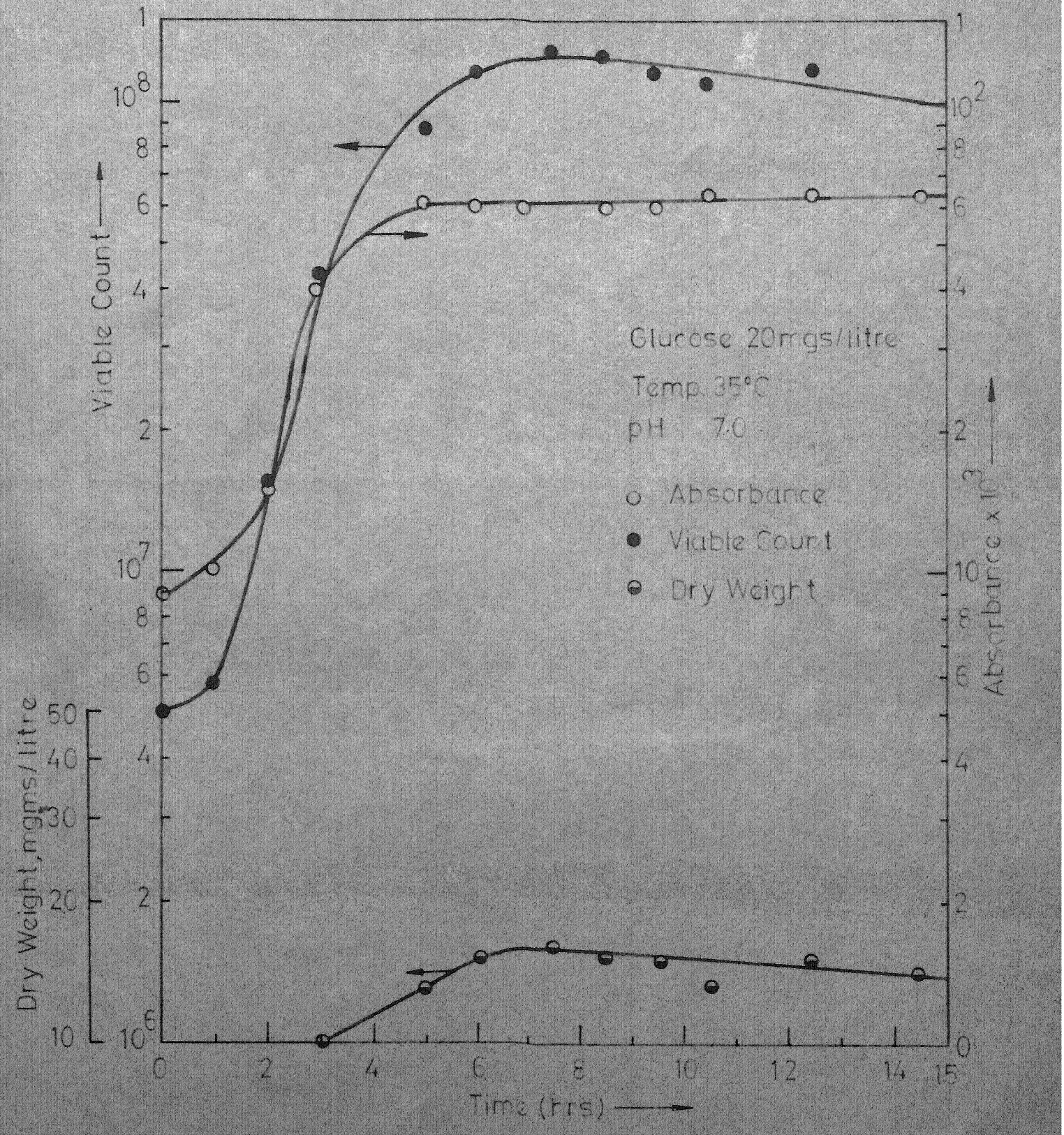

Fig. 3 - Batch Culture.

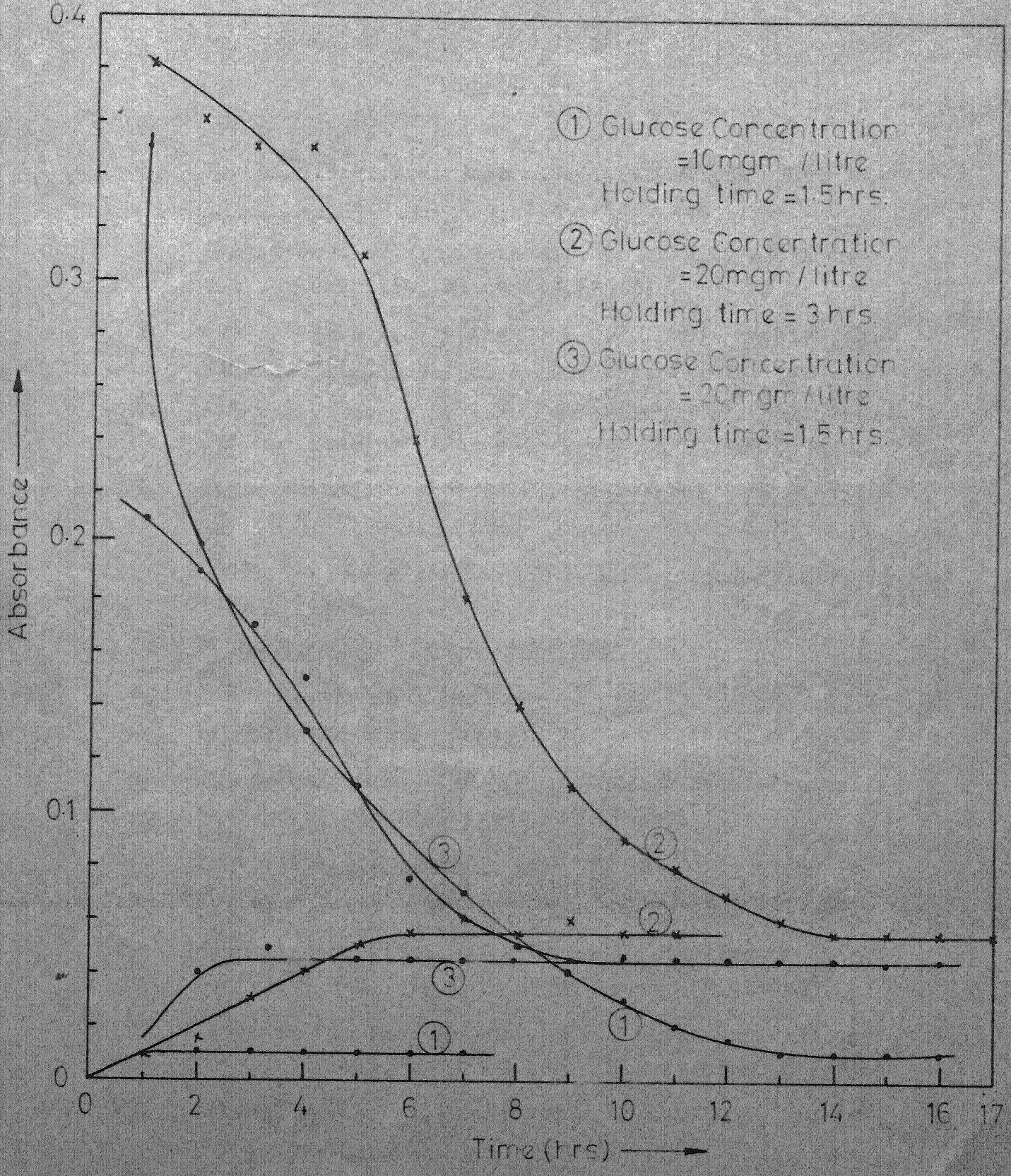

Fig. 4 – Continuous Culture.

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APPENDIX - A.

MEASUREMENTS OF GROWTH PARAMETERS

1. TURBIDITY :

Turbidity of samples was measured at 420 mμ on "Spectronic 20". Turbidity is not a linear function of dry weight or cell number at higher values (23). Therefore the samples were suitably diluted so that its turbidity fall within the range from o to 0.4.

2. DRY WEIGHT :

100 mls of culture was centrifuged in a high speed centrifuge (sorvall, ss-3) at 5000 RPM for 10 minutes. The supernatant was taken out by suction using a water air suction pump. The bacterial mass was resuspended in distilled water and centrifuged again. The cells were in this way washed and centrifuged till all the salts of solution were completely removed. This was tested by testing for Chloride in supernatant by silver nitrate solution. The cell suspension then was transferred to small weighing tubes and dried in an oven set at 85°C till it gave constant weight of cells. The weighing tubes were cooled down to room temperature by keeping them in a desiccator for 5 minutes before weighing.

3. VIABLE COUNT :

Viable count gives the number of viable cells in culture. A.014M NaCl solution was used for serial dilution as this strength of NaCl solution exert favourable action on the viability of E.coli (24). Plate count method (25) was used in this work for viable count. Cotton plugged test tubes containing 9 ml., of saline sterilized in autoclave were used for dilution. A Colony counter was used for counting the colonies.

4. GLUCOSE ESTIMATION :

Glucose concentration was estimated by using Folin and Wu(26) method. The culture samples were centrifuged at

5000 r.p.m. for 10 minutes and supernatant was taken as a sample for glucose estimation. The colour developed by phosphomolybdic acid colour reagent was measured by using "Spectronic 20" at 500 mμ. A blank and a standard solution were run with each set of samples analysed.